Paraoxonase 2 Acts as a Quorum Sensing–Quenching Factor in Human Keratinocytes

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TO THE EDITOR

Many pathogenic bacteria are characterized by their ability to express virulence factors such as proteases, toxins, and biofilms. The expression of several virulence factors is controlled by quorum sensing (QS), a bacterial cell-to-cell signaling system. QS is mediated by the production of small signal molecules termed autoinducers, which can activate or repress gene expression upon reaching a certain minimal threshold concentration (Antunes *et al.*, 2010). In many Gram-negative pathogens including *Pseudomonas aeruginosa*, *N*-acyl homoserine lactones (AHLs) are used as autoinducers to regulate the expression of virulence factors (Galloway *et al.*, 2011). AHLs can be degraded by lactonases, a process termed quorum quenching (Zhang and Dong, 2004). Production of AHL-degrading enzymes appears to be an effective strategy to interfere with bacterial cell communication and to hamper the expression of virulence factors.

Paraoxonase (PON)1–3 are three human lactonases originally described as enzymes that are capable of hydrolyzing organophosphates (Precourt *et al.*, 2011). Although much research interest has been focused on the antioxidative and anti-inflammatory properties of PON1–3 (Precourt *et al.*, 2011), the lactonase activity of these enzymes led to the hypothesis that they may also have a role in attenuation of bacterial virulence through interfering with QS (Camps *et al.*, 2011). In line with this hypothesis, it has been shown that murine tracheal epithelial cells express PON1–3, and loss of PON2 in murine PON2-deficient tracheal epithelia cultures enhanced *P. aeruginosa* QS (Stoltz *et al.*, 2007). Overexpression of PON2 increased AHL inactivation in human airway epithelial cell lysates (Stoltz *et al.*, 2007), and PON2 small interfering RNA (siRNA)–treated hepatoma and endothelial cell line lysates showed a decreased capacity to hydrolyse *N*-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL), one of the major AHLs produced by *P. aeruginosa* (Teiber *et al.*, 2008). In another study, it was shown that PON1-overexpressing transgenic *Drosophila* was protected from *P. aeruginosa* lethality and that protection was dependent on the activity of the PON1 lactonase (Stoltz *et al.*, 2008). Together, these studies suggest that PONs have an important role in the quenching of bacterial QS.

As nothing is known about the capacity of human skin to interfere with QS, we sought to investigate whether PONs are functionally expressed in human keratinocytes. First, we analyzed gene expression of PON1–3 in human primary keratinocytes using real-time PCR. Plasmids containing inserts with the PON1–3 PCR products served as standards for absolute quantification of PON transcripts. As shown in Figure 1, we detected high levels of *PON2* gene expression in primary keratinocytes, whereas PON3 was less expressed and PON1 was below the level of detection (Figure 1a). This is in line with a recent report in which a PON2 but not a PON1 band in gel-based PCR of skin extract was detected (Mackness *et al.*, 2010). In addition, we detected no induction of *PON2* gene expression in keratinocytes stimulated with various cytokines, growth factors, and *P. aeruginosa* (Figure 1b and c). Protein expression of PON2 was analyzed by immunohistochemistry with PON2 antibodies using paraffin sections of normal and psoriatic skin. Strongest PON2 immunoreactivity was detected in the outermost epidermal layers of normal and psoriatic skin (Supplementary Figure S1 online). We detected no obvious differences in the staining intensities between normal and psoriatic skin. In line with these data, a real-time PCR analysis revealed no significant differences in *PON2* gene expression between three lesional and non-lesional psoriasis samples (not shown).

It has been shown that of the three PON enzymes PON2 exhibits the highest activity toward the important AHL 3OC12-HSL (Teiber *et al.*, 2008). To assess whether 3OC12-HSL is able to induce PON2 expression, we stimulated keratinocytes with different concentrations of 3OC12-HSL for 3 and 16 hours. 3OC12-HSL was not able to induce *PON2* gene expression in keratinocytes (Figure 1d).

The high expression level of PON2 in keratinocytes together with its strong lactonase activity suggests that PON2 may have a role in cutaneous defence against bacterial pathogens through interference with bacterial QS by signal molecule inactivation. To verify the functional relevance of PON2 expression in keratinocytes, we incubated primary keratinocytes with 20 μM 3OC12-HSL. Following 3 hours of incubation, the medium was removed and analyzed for the presence of 3OC12-HSL by the use of the biosensor strain *Pseudomonas putida* F117 (pAS-C8), which expresses green fluorescent protein (GFP) in the presence of AHLs with acyl side chains ranging from C8 to C12 (Steidle *et al.*, 2001). A 50-μl

Abbreviations: AHLs, N-acyl homoserine lactones; GFP, green fluorescent protein; 3OC12-HSL, N-(3-oxododecanoyl)-L-homoserine lactone; PON1–3, paraoxonase 1–3; siRNA, small interfering RNA; QS, quorum sensing

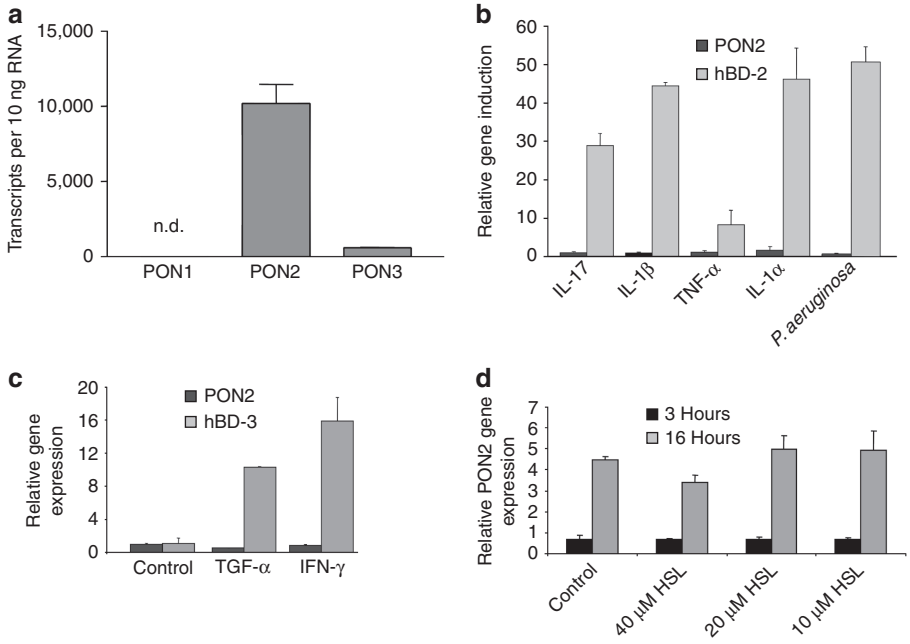

Figure 1. Gene expression of paraoxonase 2 (PON2) in human keratinocytes. (**a**) Transcript levels quantified by real-time PCR of PON1–3 in primary keratinocytes. Absolute transcript levels are shown per 10 ng total RNA (n.d. = not detectable). (**b**, **c**) Primary human keratinocytes were stimulated for 16 hours with the indicated cytokines and growth factors (each 20 ng ml^{-1}), as well as with living *Pseudomonas aeruginosa* (strain PAO1, 4×10^6 per ml), and relative gene expression of PON2, as well as hBD-2 and hBD-3 (serving as positive control), was analyzed using real-time PCR and normalized to expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (**d**) Primary keratinocytes were stimulated for 3 and 16 hours with the indicated concentrations of *N*-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL). Relative *PON2* gene expression was analyzed by real-time PCR and normalized to the expression of the housekeeping gene GAPDH. Data represent the means ± SD of three stimulations. TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

sample of keratinocyte supernatant was added to 50 µl of the sensor strain and GFP fluorescence was measured at 485 nm (extinction) and 520 nm (emission). A standard curve with different concentrations of 3OC12-HSL was generated and used for quantification. 3OC12-HSL incubated in a medium without keratinocytes and keratinocytes in the absence of 3OC12-HSL served as controls. Incubation of 3OC12-HSL for 3 hours with keratinocytes resulted in a strong reduction of 3OC12-HSL activity as compared with the control without cells (Figure 2a). 3OC12-HSL was not inactivated when incubated with keratinocyte culture supernatants alone, indicating that contact with the cells is necessary for inactivation of the signal molecule (Figure 2a). This is in concordance with the observation of Chun *et al.*, 2004, who reported that 3OC12-HSL was inactivated in airway epithelial cells by a cell-associated activity rather than a secreted factor.

To assess whether PON2 contributes to the capability of keratinocytes to inactivate 3OC12-HSL, we conducted these experiments with keratinocytes treated with PON2-specific siRNA to downregulate the expression of PON2. Real-time PCR analyses revealed that exposure of keratinocytes to PON2 siRNA (Qiagen, Hilden, Germany) resulted in an approximately 90% reducztion of *PON2* gene expression as compared with keratinocytes treated with a control siRNA (allstars control, Qiagen; Figure 2b). Keratinocytes transfected with PON2 siRNA showed a significant decrease in their capacity to inactivate 3OC12-HSL (Figure 2c). Similar results were obtained using another siRNA specific for PON2 (not shown). These data indicate that PON2 contributes to the 3OC12-HSL-degrading activity of primary keratinocytes.

To further assess the physiological relevance of PON2-mediated interference with *P. aeruginosa*, we incubated living *P. aeruginosa* for 5 hours with PON2 and control siRNA–transfected primary keratinocytes. Real-time PCR analysis of *P. aeruginosa* gene expression after incubation with PON2-downregulated keratinocytes revealed a weak induction of the QS-regulated virulence factor, lasB elastase, and the rhamnolipid biosynthesis gene, *rhlA* (Pearson *et al.*, 1997; 1.6-fold and 1.9-fold, respectively; data not shown).

As quorum sensing has a role in bacterial adhesion, we used a keratinocyte adhesion assay to study the interaction of *P. aeruginosa* with keratinocytes (Wang *et al.*, 2005). This revealed that keratinocytes transfected with PON2 siRNA were significantly more colonized with *P. aeruginosa* than the control cells (Figure 2d). These data indicate a role of PON2 in influencing the colonization of keratinocytes with *P. aeruginosa*.

In addition, *P. aeruginosa*-mediated induction of IL-1β and IL-8 was decreased in the PON2-downregulated keratinocytes (Figure 2e and f). Although further experiments have to elucidate the molecular mechanisms, it is possible that increased levels of HSL molecules upon downregulation of PON2 may negatively influence cytokine

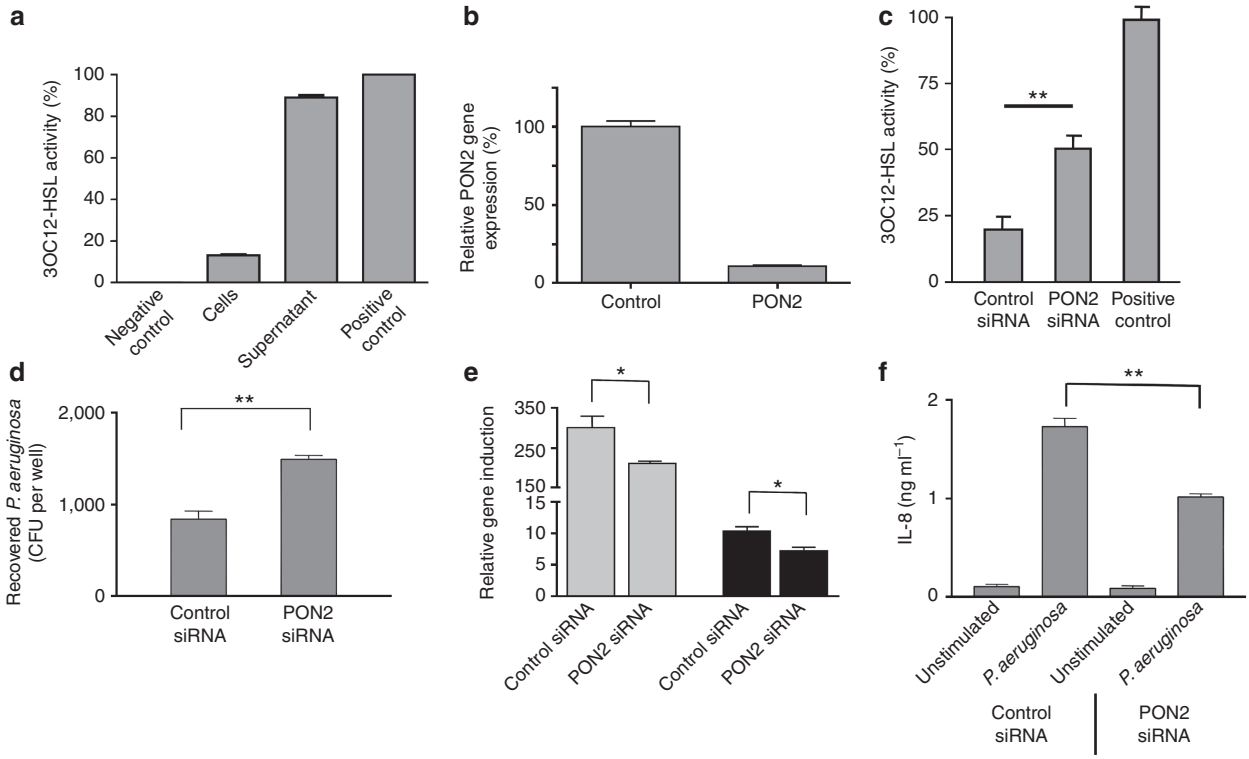

Figure 2. Paraoxonase 2 (PON2) mediates inactivation of the bacterial signal molecule *N*-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL) by human keratinocytes. (**a**) Primary human keratinocytes were incubated with 20 μM 3OC12-HSL for 3 hours before the amount of remaining 3OC12-HSL was determined using the *N*-acyl homoserine lactone (AHL) biosensor *Pseudomonas putida* F117(pAS-C8). Cells: incubation of 3OC12-HSL with keratinocytes; supernatant: incubation of 3OC12-HSL with keratinocyte supernatant without cells; negative control: keratinocyte culture medium without 3OC12-HSL; positive control: incubation of 3OC12-HSL in keratinocyte culture medium. (**b**) The knockdown efficiency of primary keratinocytes transfected for 48 hours with 15 nM PON2-specific small interfering RNA (siRNA; Qiagen) or unrelated control siRNA (allstars siRNA, Qiagen) was assessed by analyzing *PON2* gene expression normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression using real-time PCR. (**c**) PON2 and control siRNA-transfected primary keratinocytes were incubated with 20 μM 3OC12-HSL for 3 hours before the remaining concentration of 3OC12-HSL in the keratinocytes culture supernatants was determined. Keratinocyte culture medium incubated with 20 μM 3OC12-HSL served as positive control. A standard curve with different concentrations of 3OC12-HSL was generated for quantification of 3OC12-HSL. Data show means ± SD of triplicate samples of one experiment representative of three independent experiments using primary keratinocytes derived from different donors. Student's *t*-test was used for statistics (**$P<0.01$). (**d**) PON2 and control siRNA–transfected primary keratinocytes were differentiated for 48 hours with 1.7 mM $CaCl_2$ and incubated for 3 hours with a living clinical isolate of *Pseudomonas aeruginosa* (1.5×10^3 CFU per 2 cm^2 well). Subsequently, adherent bacteria were analyzed after washing and trypsination of keratinocytes and plating on agar plates as recently described (Wang *et al.*, 2005). A representative experiment of three independent experiments conducted in triplicates is shown. **$P<0.01$, Student's *t*-test. (**e**, **f**) PON2 and control siRNA–transfected primary keratinocytes were stimulated for 5 hours with living *P. aeruginosa* (PAO1, 6×10^7 per ml). Subsequently, RNA was isolated and reverse transcribed in complementary DNA (cDNA), which served as a template in a real-time PCR to determine *IL-8* and *IL-1β* gene expression normalized to GAPDH expression. The relative *P. aeruginosa*–mediated gene induction of IL-8 (gray bars) and IL-1β (black bars) in keratinocytes transfected with control or PON2 siRNA is shown (**e**). In addition, culture supernatants were analyzed using an IL-8 ELISA (R&D Systems, Minneapolis, MN) (**f**). Data show means ± SD of triplicate samples of one experiment representative of three independent experiments using primary keratinocytes derived from different donors. Student's *t*-test was used for statistics (*$P<0.05$, **$P<0.01$).

expression because it has been reported that 3OC12-HSL is able to negatively regulate the expression of proinflammatory cytokines through interference with NF-kappaB (Kravchenko *et al.*, 2008).

In summary, this study demonstrates that keratinocytes are able to inactivate the signal molecule 3OC12-HSL and that this activity is partly mediated by PON2. Thus, PON2 acts as a QS-quenching factor in keratinocytes and may have an important role in cutaneous defense against bacterial infections.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Modeling Epidermal Melanoma in Mice: Moving into New Realms but with Unexpected Complexities

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TO THE EDITOR

Histological localization of human and murine melanomas is very different. Most human melanomas are epidermal, invading the dermis during progression, whereas murine lesions are usually exclusively dermal. We recently took a mouse model of dermal melanoma (*Arf*$^{-/-}$*::Tyr-NRAS*^{Q61K}), in which melanocytes are rarely seen in the epidermis of normal or lesional skin, and combined it with an animal carrying epidermal melanocytes throughout life due to the overexpression of the Kit receptor ligand in keratinocytes (*K14-Kitl*). *Arf*$^{-/-}$*::Tyr-NRAS*^{Q61K}*::K14-Kitl* mice developed slow-growing plaques exhibiting a pagetoid growth pattern typical of human superficial spreading melanoma (SSM), and our experiments suggested a probable epidermal origin (Walker *et al.*, 2011a). In contrast, Rae *et al.* (2012) report the development of rapidly growing dermal melanomas in *Braf*^{V600E}*::K14-Kitl* mice, which show no epidermal involvement. As *BRAF*^{V600E} is the most common mutation in SSM, modeling *Braf*^{V600E}-driven epidermal melanomas in mice would be an important advance. We propose that this failure to induce epidermal melanoma is unlikely to be a particular feature of Braf^{V600E}-driven murine melanoma, and that the contrasting results illuminate critical aspects of experimental design to be considered when making mechanistic inferences about particular engineered mutations. Rae *et al.*, outlined these differences: (1) strain background, (2) use of NRAS^{Q61K} versus Braf^{V600E}, (3) we used neonatal UV radiation (UVR), (4) we used an additional Arf mutation, and (5) different timing of mutation induction. Given the importance of generating epidermal melanoma in mice for their utility as preclinical models, we have examined these differences in detail.

Strain background can influence penetrance and other aspects of neoplasia, but as epidermal melanocytes are present in both studies it alone seems unlikely to explain the contrasting propensities for epidermal involvement. In addition, we know of no evidence in the melanoma field that would lead us to suspect that NRAS^{Q61K} and BRAF^{V600E} would behave differently in this context. In a spontaneous melanoma model (*Cdk4*$^{R24C/R24C}$*::Tyr-NRAS*^{Q61K}*::K14-Kitl*), we observed many atypical epidermal melanocytes (Figure 1a) and the mice developed plaque-like lesions with atypical melanocytes scattered throughout the epidermis (Walker *et al.*, 2011b and Figure 1b). Hence, neonatal UVR is not obligatory for epidermal melanoma genesis in *K14-Kitl* mice. We also examined the consequence of NRAS^{Q61K} expression on melanocyte proliferation in *K14-Kitl* mice without accompanying Arf or Cdk4 mutation. In *Tyr-NRAS*^{Q61K}*::K14-Kitl* neonates, we observed a striking increase in epidermal and upper follicular melanocytes compared with mice carrying *Tyr-Nras*^{Q61K} or *K14-Kitl* alone (Figure 1c). In adult *Tyr-NRAS*^{Q61K}*::K14-Kitl* skin, melanocytes were frequently almost at confluence in the basal epidermis, exhibiting variation in nuclear size and shape, nesting, and suprabasal localization (Figure 2a and b) (we did not perform long term studies on melanoma development for this genotype). NRAS^{Q61K} expression greatly

Abbreviations: mGluR1, metabotropic glutamate receptor-1; SSM, superficial spreading melanoma; UVR, UV radiation